SEPARATION OF PROTEIN X FROM THE DIHYDROLIPOYL TRANSACETYLASE COMPONENT OF THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX AND THE STUDY OF PROTEIN X

by

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TO MY MOTHER AND FATHER
WITHOUT THEIR FINANCIAL AND EMOTIONAL SUPPORT
I COULD NOT HAVE ACHEIVED WHAT I HAVE

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INTRODUCTION

Pyruvate dehydrogenase complex (PDC) occupies a key position in intermediate metabolism by oxidatively decarboxylating pyruvate to acetyl-CoA, an irreversible step in the utilization of carbohydrate. PDC is a classic example of a multienzyme complex. As isolated from most sources, three distinct components function in the overall reaction. The pyruvate dehydrogenase component of the complex, termed E1, uses thiamin pyrophosphate as an essential cofactor. Dihydrolipoyl transacetylase component, designated E2 has a lipoic acid cofactor attached to it. Dihydrolipoyl dehydrogenase is designated E3 and contains an FAD cofactor.

The E2 component of the complex has three major roles:(1) it forms the central core around which are arranged multiple copies of the E1 and E3 components,(2) it provides attachment sites for the lipoic acid cofactor,and (3) it catalyzes the reaction which forms the acetyl-CoA product [1]. The E2 subunits of the mammalian complex are arranged as a pentagonal dodecahedron which has 532 symmetry with 60 copies of the dihydrolipoyl transacetylase component.

E2 has several functional domains which includes a compact inner domain and an extended outer domain. The inner domain contains the active site which catalyzes the transacetylase reaction. One or more outer domains containing a lysine group with a

lipoic acid group attached via a thioester linkage is present in different E2's. The lipoic group region moves between the active sites of the E1, E2 (transacetylase) and E3 components. Between the inner domain and extended outer domains there is a region called the binding domain. In E. coli this distinctive region is thought to bind the E3 component to the complex [3]. The three domains are joined by linker regions shown to be high in proline and alanine giving them increased flexibility [4]. This is analogous to mammalian PDC where the linker region sequence has been derived but the function has not been proven.

E1 and E3 components are less complicated enzymes in structure relative to E2. E3 is an FAD containing flavoprotein and contains a disulfide bridge which undergoes oxidation and reduction during catalysis. The Porcine heart E3 sequence has been derived and the subunit has 49,690 Mr [5]. E1 is a tetramer consisting of 2 non-identical subunits; α and β.

The kinase and phosphatase acting on PDC are specific to the complex [6]. The kinase subunits are tightly bound to and copurify with PDC while the PDC phosphatase is loosely associated and is purified as a distinct soluble enzyme [7]. The kinase consists of two non identical subunits termed K_c and K_b and is apparently a dimer [7]. The K_c subunit (46,00 Mr) contains the kinase active site and the K_b subunit (43,000 Mr) is thought to be regulatory. The bovine PDC phosphatase consists of two subunits with the catalytic activity residing in the smaller (49,000 Mr). The larger

subunit (89,000 Mr) contains an FAD cofactor [8] but the function of this has not been determined.

In mammalian PDC, there is a component identified as protein X [9][10] which is tightly bound to E2. Initially it was assumed [2] that protein X was a proteolytic fragment of E2 but evidence has shown that protein X is a distinct component [9][10]. Protein X contains at least one lipoyl moiety which like E2 lipoyl groups can be reductively acetylated [9]. It was suggested that protein X is responsible for binding PDC kinase but this was not firmly established.

Since it was not clear how protein X is bound to the core, I hoped to gain some knowledge of this by looking how protein X separates from the core. I wished to develope techniques to remove protein X away from the E2 core and then to evaluate the properties of the E2 core that is lacking in protein X and if possible totally free of protein X. I hoped to learn how the removal of protein X affects the activity of the E2 core enzyme and how it affects the PDC complex as a whole.

EXPERIMENTAL PROCEDURES

Materials. Highly purified bovine kidney pyruvate dehydrogenase complex (15-18 micromole/mg/min) was prepared by the procedure developed by Roche and Cate (1977) [11]. The E2-X-K_cK_b and E1 components were prepared under the procedure of Linn et al [6]. The E2-X_I was prepared by selectively removing the lipoyl bearing domain of protein X by treatment with protease Arg C [12]. Porcine heart E3 was purchased from Sigma Chemical Company. Bovine serum albumin (BSA), used as a protein standard, was purchased from U.S. Biochemical Corp. [3-^{14}C]pyruvate and [1-^{14}C]acetyl-CoA were purchased from New England Nuclear, Du Pont Co. All other materials were of the highest grade possible.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted using the Laemmli System.[18] Staining was with Coomassie Blue G-250 formulated under 3.11.3 of Neuhoff et al.

PDC Reconstitution Assay. The NAD^+ reduction activity of the pyruvate dehydrogenase complex was determined at 30°C by monitoring the increase in absorbanceat 340 nm [6]. The assay mixture consisted of 50 mM potassium phosphate buffer pH 7.3-7.5, 0.2 mM thiamin pyrophosphate, 1.0 mM of Coenzyme A, 2.6 mM cysteine hydrochloride, 2.0 mM of pyruvate and enzyme. For assaying

reconstituted complex, the incubation mixture included 1.0 µg E2 source was incubated with 0.5 µg E3 (dihydrolipoyl dehydrogenase component) and 1.0 µg E1 (pyruvate dehydrogenase component) in a total of 15 µl 50 mM potassium phosphate buffer for 5 min at 4 C before adding 10 µl to the assay cuvette. Activity was expressed as µmoles of NADH formed per minute per mg of enzyme. The E2-X-K_cK_b subcomplex was used as an E2 and X source in control assays.

E1 acetylation assay. Acetylation of the lipoyl moieties of E2 by [3-^{14}C]pyruvate was measured at 4° and 22° C [9]. At 4°C, E1 and E2 were incubated for 5 minutes with 125 µM TPP and 1.0 mM $MgCl_2$ and diluted 5 fold into reaction mixture with final concentrations of 250 µM [3-^{14}C] pyruvate, 25 µM TPP , 200 µM $MgCl_2$ and 50 mM K_xPO_4 pH 7.3. At 22°C all conditions were kept the same except E1 and E2 were preincubated with 12.5 mM TPP prior to dilution into assay mixture. The final concentration of TPP was 2.5 µM. At timed intervals samples were removed and spotted on TCA soaked discs which were subsequently washed 4X with 10% TCA (trichloroacetic acid) for 30 minutes followed by two 5 minute washes with absolute ethanol and two 3 minute washes with ether under hood. Discs were placed in scintillation fluid and protein bound radioactivity was measured.

Non-radioactive transacetylation assay. This assay was used as a rapid method for determining activity during resolution steps. Dihydrolipoyl transacetylase (E2) transfers the acetyl group of

acetyl-CoA to dihydrolipoamide and the S-acetyldihydrolipoamide (S-acetyl DHL) product is measured spectrally at 232 nm. Activity is measured in the presence of a low level of acetyl-CoA that is constantly being regenerated by a reaction transferring the acetyl group from acetyl phosphate to CoA which is catalyzed by phosphotransacetylase (PTA). The reaction mixture consists of 1-5 µl of PTA (1,000 units per ml), 30 mM TRIS-HCl pH 7.5, 1.0 mM dihydrolipoamide (DHL), 1.0 mM acetylphosphate, 5 mM cysteine, and 5 mM CoA in a total volume of 1 ml. The reaction mixture without addition of enzyme proceeds until there is no further increase in A_{232} due to the formation of acetyl-CoA and then E2 source is added. The increase in absorbance at 232 nm due to formation of S-acetyl DHL is measured and the activity reported as ΔOD/min.

Radioactive dihydrolipoyl transacetylase assay. Radioactive S-acetyl DHL formed from reaction of [1-^{14}C]acetyl-CoA with DHL was extracted into benzene leaving the unreacted in the aqueous phase [14]. The reaction mixture consisted of 0.25 ml 50 mM potassium phosphate pH 7.4, 0.1 ml of 2.5 mM DHL in 25% ethanol, 0.1 ml of 2.5 mM [1-^{14}C]acetyl-CoA (~700 cpm/pmol),and 4-10 µg of E2 source. Deionized water was added to make the total volume of the assay 0.5 ml. The reaction was started by the addition of the [1-^{14}C]acetyl-CoA and incubated at room temperature for 2 min. 1 ml of cold benzene was added and vortexed for 30 sec to stop the reaction and to initiate the extraction of the S-acetyldihydrolipoamide. All

sample were again vortexed and then centrifuged at room temperature in a clinical centrifuge at 1500 rpm for 5 min to separate layers. 0.2 ml of the benzene phase was withdrawn and radioactivity measured in a Beckman liquid scintillation counter. Control differed by lacking the addition of enzyme. Activity was expressed in μmoles/min/mg.

Binding of E1 and E3 components to E2 source. In 110 μl of 50 mM sodium phosphate buffer pH 7.5 containing 0.2 mM EDTA and 5 mM DTT, 30 μg of E1 was incubated with three different E2 sources (E2–X–K_cK_b, E2–X_I, and E2 oligomer) for 10 min. at 4° C [15]. Control samples consisted of each E2 source alone and 30 μg of E1 alone. The entire sample was layered over a three step sucrose gradient (20 μl each of 7.5%, 10% and 15% sucrose dissolved in the same buffer). The samples were centrifuged in a rotor, tube and adaptor in a Beckman Ultracentrifuge at 130,000 g for 120 min. The upper 150 μl was removed and designated the first supernatant. The rest of the supernatant was removed and designated the second supernatant. The pellet was dissolved in 50 μl of sample buffer and SDS PAGE was conducted using 15 μl of 1st supernatant and 5 μl of pellet. The gel was stained with Coommassie Blue stain and photographed.

Conditions for evaluating E3 binding to the same E2 core were the same as those used for E1 above except only 10 μg of E3 was incubated with the E2 source and the centrifugation time was

extended to 180 min at 130,000g.

O-phthaldehyde (OPA) protein measurement. The protein concentration of each pellet and supernatant was determined by the procedure of Fried et al (1984) [20] using fluorescence of OPA reacting with the available amino and thiol groups of the protein. This sensitive assay system combines the OPA derivatization procedures and the separation of fluorescent protein-SDS complexes from the interfering fluorescence of small molecular weight products using a 10 inch size exclusion TSK columns. Excitation and emission wavelengths were 330 nm and 418 nm, respectively. The assay was linear over the range of 3 ng to 1 µg of protein. For our purposes, a BSA standard curve was used in the range of 0.2 µg up to 1.0 µg.

BCA protein measurement. A set of BSA protein standards were prepared in 50 mM phosphate buffer pH 7.5 ranging from 0.25 µg to 2 µg per 10 µl. 10.0 µl of each standard (final µg ranging from 0.25 µg to 2.0 µg) and sample were placed into wells of a 96 well ELISA assay plate [16]. The working reagent was prepared by mixing Reagent A with Reagent B in a ratio of 50:1. 200 µl of working reagent was added to each well. Blanks consisted only of buffer and working reagent. The plate was covered and incubated at 60° C for 30 minutes, cooled and absorbance read at 590 nm.

Microplate Binding Assay. Samples of E2-X subcomplex (or other

fractions to be tested for binding) were incubated in 100 µl of phosphate buffered saline (PBS: 8 mM Na_2HPO_4, 1.15 mM KH_2PO_4, 2.6 mM KCl, 136 mM NaCl, and 0.2 mM EDTA) for 14 hours at 6° C in the wells of a 96 well ELISA plate [19]. Following 4 washes with 100 µl PBS, the level of protein bound was measured in quadruplicate using Pierce BCA [16]. A parallel set of wells treated with 200 µl of PBS containing 1% (w/v) ovalbumin for 4 h. Following two washes with PBS, 2.0 µg of E3 in 100 µl PBS containing 1 mg/ml ovalbumin was added to the wells and incubated for 40 minutes at 23°C. Control wells were blocked with 1% (w/v) ovalbumin in PBS and treated with the 2.0 µg E3 component. Two rapid washes with PBS containing 1 mg/ml ovalbumin were performed and then dihydrolipoyl dehydrogenase activity measured by adding 200 µl of 50 mM potassium phosphate (pH 7.5) containing 1.0 mM NAD^+, 1.0 mM dihydrolipoamide, and 0.5 mM EDTA. At the indicated time, 190 µl aliquots were removed and the reaction quenched by the addition of 190 µl of 2M guanidine·HCl in 50 mM potassium phosphate pH 7.5 at 4° C and thoroughly mixed. The absorbance at 340 nm was measured in a Beckman DU 8 spectrometer.

RESULTS

Initial E2 oligomer resolution

E2-X-K_cK_b is composed of ~ 60 E2 subunits (associated to form a pentagonal dodecahedron), ~6 protein X subunits and 2-3 PDH kinases dimers. The E2-X-K_cK_b subcomplex was incubated in various concentrations of urea and NaCl for different lengths of time. The objective was to try to separate an oligomeric E2 component from protein X and the K_cK_b components. This treated subcomplex was then applied to a 0.7 x 45 cm Sephacryl S-300 or a S-400 gel filtration column equilibrated with the same level of chaotropic reagents. The protein content of the fractions collected from the column was determined by the absorbance at 280 nm was measured using the column buffer as a blank. Dialysis was conducted, protein concentration was determined and SDS-PAGE conducted to estimate the extent of separation of protein X away from the E2 component.

The initial resolution attempts were conducted using 2 M and 5 M concentrations of urea in 50 mM sodium phosphate buffer pH 7.4 containing 0.5 mM DTT. The period of incubation in these buffers was 30 minutes at 4° C prior to the gel filtration step. The eluent buffer on the column was the same as the incubation urea buffer except it contained 0.2 mM EDTA as well. The fractions collected from the 2 M urea column showed little separation between components. The 5 M urea column showed significant separation of

protein X away from the E2 core. The later (downstream) fractions showed an increased ratio of X to E2 compared to the standard E2–X–K subcomplex (Figure 2).

In order to increase the resolution of protein X from the E2 core, the urea concentration was increased to 6 M and the gel filtration was again conducted. SDS–PAGE showed only a trace amount of X appeared in the upstream fractions that contained E2 (Figure 3). Protein measurements were performed using the OPA method. Spectrophotometric assays were performed using E2–X–K_cK^b as a standard. The E2 activity (S–acetyl–DHL measured at 232 nm) of the fractions containing low ratios of protein X vs. E2 had substantially decreased the specific activity for E2. The yield off the column was less than 50% of the initial protein used in the incubation.

A study of the effects on the E2 activity of E2–X–K_cK_b subcomplex following the treatment with various concentrations of urea was performed. Treatment with 5 M urea decreased E2 specific activity 10% vs. untreated E2–X–K_cK_b subcomplex. However, a large decline in E2 specific activity was caused by the treatment in 6 M urea, probably due to the unfolding of the E2 subunits. Compromising urea concentration levels were picked at 5.5 M and an additional pH variable was added. Two sets of resolutions were performed using pH 6.5 and pH 8.5 both using 5.5 M urea concentrations. Separation of protein X from E2 was not complete and transacetylation specific activity held steady with that of E2–X–K_cK_b.

Modifications in resolution procedure.

Variations in resolutions using the same urea concentration caused a re-evaluation of the resolution variables. Further studies concluded that urea concentrations were optimal at 5.5 M. Anything higher caused a decrease in transacetylation specific activity. Concentrations lower than 5.0 M urea decreased the efficiency of the resolution. In an attempt to keep the separation high without decreasing transacetylation activity, the column matrix was replaced with Sephacryl S-400 and 0.5 M NaCl was added to the incubation buffer and column buffer while the urea concentration was held at 5.5 M.

With the addition of the NaCl to the resolution protocol, the separation of protein X from the E2 fraction was greatly improved. Transacetylation specific activity of the resolved E2 remained comparable with E2-X-K_cK_b subcomplex specific activity. E2 fractions with little or no protein X were combined and the oligomeric core pelleted together. E2 fractions with increased levels of protein X were also combined and pelleted. The purpose of the centrifuging the fractions was to bring down into the pellet the oligomeric assembled units and more native E2 components while leaving the unfolded or non native components in the supernatant. Reconstitution PDC activity assays were performed. The PDC activity of the E2 oligomer freed of most of the protein X was only 20% of that found from E2-X-K_cK_b starting material. Thus while the

transacetylation specific activity had not decreased, either the lack of protein X or the structural modifications of E2 resulting from the resolution process were limiting the reconstitutional PDC activity.

In addition, the yield from the treatment of 1 mg E2-X-K_cK_b was quite low with approximately 400 µg of protein eluting from the column and only 100 µg of the E2 oligomer freed of protein X was recovered.

Addition of Pluronic F-68.

Pluronic F-68, a block polymer with an average composition of $H(OCH_2CH_2)_{75}-(OCH(CH_3)CH_2)_{30}-(OCH_2CH_2O)_{75}H$, was tested to see if it would improve the yield off the column. The basic concept behind the use of Pluronic F-68 was that the hydrophobic center of the block polymer would interact with the exposed hydrophobic regions of the enzyme components preventing the E2-X-K_cK_b from interacting with the hydrophobic areas of the column matrix and therefore increase the protein yield off the column. PDC pretreated with Pluronic F-68 was evaluated for retention of activity and found that there was no effect on PDC when incubated with different levels of Pluronic F-68 in the range of 0.5 mg/ml up to 10 mg/ml.

A resolution of E2-X-K_cK_b was conducted with 5.3 M urea and 0.35 M NaCl in which Pluronic F-68 was added to both the incubation mixture and the elution buffer at a concentration of 1 mg/ml. The results of this addition was to decrease in separation of the

protein X from the E2. Another resolution was then conducted in which Pluronic F-68 was added only to the column buffer at a concentration of 0.2 mg/ml with no Pluronic F-68 added to the incubation mixture (Figure 4). This gave the best results. Protein recovery increased to close to 700 µg of total protein from the column when starting material was 1 mg E2-X-K_cK_b (Table 1). About 250 µg of protein was recovered in the E2 pellet with the addition of Pluronic F-68 in the column buffer.

There was still some variations between resolutions in amount of separation and contamination of E2 fractions with protein X. Holding the other variables (urea, NaCl, and Pluronic concentrations) constant the time of incubation was increased from 1 hour to 90 minutes up to the final time of 2 hours. By increasing the incubation time to two hours, separation of protein X from E2 was greatly improved without decreasing E2 specific activity.

Optimal resolution conditions

After all conditions were optimized, the final protocol for the pre-incubation consisted of 50 mM sodium phosphate pH 7.5, 0.5 mM DTT, 5.3 M urea, 0.35 M NaCl with 1-2 mg E2-X-K incubated for 2 hours at 4° C. Table 2 shows the summary of resolution conditions. Sucrose was added to the incubation mixture to a final concentration of 10% (w/vol) and underlayed on a Sephacryl S-400 column matrix that was equilibrated and eluted with 50 mM sodium phosphate pH 7.5, 5.3 M urea, 0.3 M NaCl, 0.2 mM EDTA, and 0.2 mg/ml Pluronic F-68.

Absorbance of each fraction was measured at 280 nm (Figure 5) and fractions with protein were dialyzed against 50 mM sodium phosphate pH 7.5, 0.5 mM DTT, 0.2 mM EDTA, and 0.1 mg/ml aprotinin (a protease inhibitor). Samples from each fraction were evaluated with on SDS-PAGE and stained with Coomasie Blue. Appropriate fractions were combined and pelleted at 190,000 g for 65 minutes. Pellets were dissolved in same buffer as used in the dialysis step. At this stage protein concentrations were determined by the BCA method and samples were frozen in liquid nitrogen and stored at -70 C. The BCA method of protein determination was preferred because it also could detect protein at the low level we needed, was less time consuming and the data were more reproducible than using the OPA protein measurement. Figure 6 shows the resolution steps from PDC to E2-X-K_cK_b subcomplex to the E2 oligomer.

Activity studies of E2 and E2X_I

Various catalytic properties of the E2 oligomer and of the E2-X_I subcomplex, prepared free of the lipoyl domain of protein X by protease arg C treatment by the procedure of Rahmattulah et al [13], were compared to those of the untreated E2-X-K_cK_b subcomplex. The E2 oligomer employed was essentially free of protein X , kinase subunits and the E3 component which originally contaminated the E2-X-K_cK_b subcomplex. Both E2 oligomer and E2-X_I had retained transacetylation specific activity. At both 4° C and 22° C, the E2 oligomer had a reduced ability to serve as an acetyl acceptor in the

reductive acetylation reaction catalyzed by the E1 component. E2-X_I subcomplex fully retained reductive acetylation activity (Table 3). The E2 oligomer and E2-X_I subcomplex both enhanced the activity of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase almost to the same extent as the E2-X (a form of subcomplex prepared from E2-X-K_cK_b in which the kinase subunits were removed) [12][17].

Both E2 and E2-X_I together with high levels of E1 and E3 components, had greatly diminished capacity to perform the overall pyruvate dehydrogenase reaction $CH_3COCO_2H + CoA-SH + NAD+ \longrightarrow CH_3CO-S-CoA + CO_2 + NADH + H^+$. This could result from denaturation of some E2 subunits in the case of E2 oligomer but that would not explain the decreased activity in the case of E2-X_I. This points to a contribution of protein X (specifically its lipoyl domains) in PDC reconstitution activity. This poses the question of the function of protein X, and how it contributes (at least in part through its lipoyl domains) to the overall reaction catalyzed by the pyruvate dehydrogenase complex. Previous studies showed that E3 selectively protected Protein X against protease action [13]. This brought into question the binding properties of E3 to E2-X_I and the E2 oligomer.

Binding properties of E2 oligomer and E2-X_I

Binding assays were performed by combining E2-X_I, E2-X-K_cK_b, and E2 oligomer with excess levels of E3 and pelleting these large E2 containing structures through a three step sucrose gradient. The idea was to see the amount of E3 bound to each of the E2 sources

after pelleting while keeping the free E3 in the supernatant. The pellets were dissolved in SDS sample buffer and run on a SDS-PAGE. Figure 7 shows that both E2 and E2-X_I had a reduced capacity to bind E3 vs. the E2-X-K_cK_b subcomplex. The inner domain of protein X may have a role but if X_I contributes it is inadequate alone. This strongly suggests the lipoyl moiety of X is needed to bind E3.

Binding assays were performed using the E1 component by combining it with E2-X_I, E2-X-K_cK_b and E2 oligomer and pelleting. In this case, E1 was retained in the pellets of all three E2 sources with no significant differences in the ratio of binding (Figure 8). This suggests that the lipoyl domain of protein X is not required to bind the E1 component to the subcomplex of pyruvate dehydrogenase complex. This also indicates that the process of preparation of the E2 oligomer did not alter the binding site on the E2 subunit for the E1 component.

Interest was then turned to the fractions in the resolution which came through the column directly after the fractions that contained only E2. These later fractions were labeled X-E2 because of the increased ratio of X to E2 compared to the E2-X-K_cK_b subcomplex. The ratios ranged from 1:2 up to 1:1 protein X to E2 as compared to 1:10 in E2-X-K_cK_b subcomplex. These levels were estimated made from the relative staining of subunits following separation by SDS-PAGE.

Transacetylation, reductive acetylation and reconstitution assays were performed on the X-E2 fractions. All activities were

greatly diminished compared to activities of the E2-X-K_cK_b subcomplex. This led us to believe that the E2 component of these X-E2 fractions was not native.

E3 binding properties of X deficient E2 sources

A microplate binding assay developed in our laboratory by S. Gopalakrishnan was utilized to evaluate E3 binding to fractions containing various levels of protein X. This included E2-X-K_cK_b, E2 oligomer, X-E2, and E2-X_I. These results (shown in Table 4) showed that the X-E2 fraction had the highest capacity of binding E3, almost three fold higher than that of E2-X-K_cK_b subcomplex (this based on the implied protein concentrations determined by the BCA protein assay). The same X-E2 fraction had reduced capacity (proportional to the E2 content) for binding ^{32}P-E1 component [19]. This again showed that protein X contributes to the binding of the E3 component.

DISCUSSION

The E2-X subcomplex associates tightly with protein X and the K_cK_b components. In order to remove these components, chaotropic agents (urea and NaCl) were used at a level high enough to separate protein X away from E2 but also to retain transacetylation activity. E2 retained the capacity to bind the El component as well as stimulate PDH kinase and phosphatase [12]. At high levels of urea and NaCl (exceeding 6.0 M and 0.5 M respectively), transacetylation activity was diminished. At levels lower than 5.0 M urea with no NaCl, the separation of protein X from E2 was not complete. In order to get maximal results in both areas, transacetylation activity and separation, exacting conditions were required.

After studying various conditions, the optimal treatment of E2-X-K_cK_b subcomplex for resolution of the E2 oligomer was found to be 0.35 M NaCl present in a 5.3 M urea buffer for both the incubation and separation by gel filtration chromatography. Without affecting the separation or transacetylation specific activity, Pluronic F-68 block polymer was added to the column buffer to increase the protein recovery from the column.

Significant native structure of the inner domain of E2 was demonstrated by the oligomer formation and transacetylation activity [13]. Other functions including El binding, kinase and phosphatase activation required the outer domains of the E2 to retain native structure [12] [13]. Decreased reductive acetylation activity was

observed and may have resulted from damage to a portion of the outer domain (specifically on the region of the lipoyl moieties that are the substrate for this reaction). The structure of the lipoyl domain is required for the reaction (free oxidized lipoamide does not serve as a substrate for this reaction catalyzed by E1).

Selective removal of the lipoyl domains of protein X by treatment of E2-X-K_cK_b with protease Arg C can be achieved without cleaving the E2 component [12]. The E2-X_I subcomplex retained transacetylation specific activity, the ability to bind the E1 component and fully activated the activities of the regulatory enzymes [12][14] and retained its capacity to serve as a substrate for the E1 component in reductive acetylation. However, the E2-X_I subcomplex had a reduced ability to perform the overall PDC reaction (Table 3). Pelleting studies also showed a reduced affinity of E2-X_I subcomplex for the E3 component. Since the removal of the lipoyl domain is what led to these latter properties, it was not surprising that I found in the same experiment that the removal of the entire protein X also reduced the affinity of the E2 oligomer for the E3 component. Removal of protein X also caused lowered activity in the overall PDC reaction although this result cannot be interpreted as clearly as in the case of the E2-X_I subcomplex since there was a reduction in the reductive acetylation reaction.

These data suggest that the E3 component of PDC associates with protein X, mainly the outer or lipoyl domain. In the binding studies, the only way that E3 could end up in the pellet is by

binding with E2–X–K_cK_b since a control was run with E3 alone and there was little or no E3 in the pellet material after centrifuging. Looking at the E2–X_I subcomplex, very little E3 was in the pellet material. Weak binding could give these results because E3 dissociates and would eventually cause it to be left behind in the step gradient pelleting procedure. The microplate binding assay detects this weak binding. Some E3 association may be due to the incomplete cleavage by arg C of the lipoyl domains (X_L) of protein X. These results point to the role of X_L portion of protein X being involved in E3 binding. Further studies are needed to show that the lipoyl domain of protein X is preferentially used by the E3 component in the dihydrolipoyl dehydrogenase reaction.

The E3 microplate binding assay also supported the association of the E3 component with protein X (Table 4). The X–E2 complex (the ratio of protein X to E2 ($\geq$1:1) being relatively high compared to E2–X–K_cK_b (1:10)) showed increased binding of the E3 component via the dihydrolipoyl dehydrogenase reaction. The E2 oligomer and E2 with low levels of protein X remaining showed lower capacities for binding the E3 component as compared to E2–X–K_cK_b. Again this suggests that increased amounts of protein X increased the capactiy to bind the E3 component. Since some E3 was bound in the microplate binding assay by the E2 oligomer and E2–X_I subcomplex, this may indicate that a domain in the E2 component (possibly through the $E2_B$ domain) contributes to the E3 binding. In some α–keto acid dehydrogenase complexes lacking a protein X, a

region of E2 subunits has been shown to contribute to the binding of the E3 component. Rahmatullah et al [12] have shown that the $E2_B$ region of the mammalian transacetylase contributes to binding the E1 component.

In summary, the evidence presented shows the association of the E3 component to protein X. Removal of protein X from the E2 component reduces the capacity of the E2 oligomer to perform the overall PDC reaction. The outer domains of protein X are required for maximal binding capacity of the E3 component and for a maximal efficiency in the reaction catalyzed by the mammalian pyruvate dehydrogenase complex.

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Figure 1. Coordinated reactions catalyzed by pyruvate dehydrogenase complex.

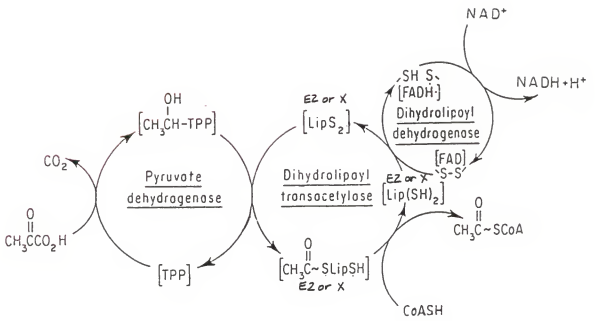
NAD+
NADH+H+
SH S
[FADH2]
Dihydrolipoyl
dehydrogenase
[FAD]
S-S
EZ or X
[Lip(SH)2]
EZ or X
[LipS2]
OH
[CH3CH-TPP]
CO2
Pyruvate
dehydrogenase
O
CH3CCO2H
[TPP]
Dihydrolipoyl
transacetylase
O
[CH3C~SLipSH]
EZ or X
O
CH3C~SCoA
CoASH

Figure 2. SDS-PAGE of E2-X-K_cK_b resolution containing 5 M urea. 1 mg E2-X-K_cK_b was incubated with 5.0M urea and 0.5 mM DTT in 50 mM Na_xPO_4 pH 7.5 for 60 minutes before applying to a gel filtration column. 0.4 ml fractions were collected and numbering started after collection of the void volume of eluent.

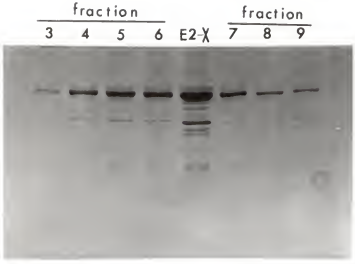
fraction
3 4 5 6
E2-X
fraction
7 8 9

Figure 3. SDS-PAGE of E2-X-K_cK_b subcomplex resolution containing 6 M urea. 1 mg E2-X-K_cK_b subcomplex was incubated with 6 M urea and 0.5 mM DTT in 50 mM Na_xPO_4 pH 7.5 for 60 minutes before applying to a gel filtration column. 0.4 ml fractions were collected and numbering began after collection of the void volume of eluent.

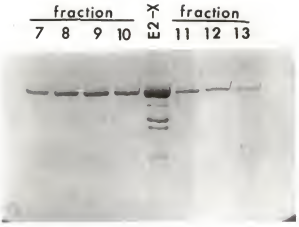
fraction
7 8 9 10
E2-X
fraction
11 12 13

Figure 4. SDS-PAGE of E2 resolution containing 5.3 M urea. 1mg E2-X-K_cK_b was incubated with 5.3 M urea, 0.35 M NaCl, 0.5 M DTT in 50 mM Na_xPO_4 pH 7.5 for 60 minutes before applying to a gel filtration column. 0.2 mg/ml Pluronics F-68 block polymer was added to column buffer only. 0.4 ml fractions were collected and numbering started after collection of the void volume.

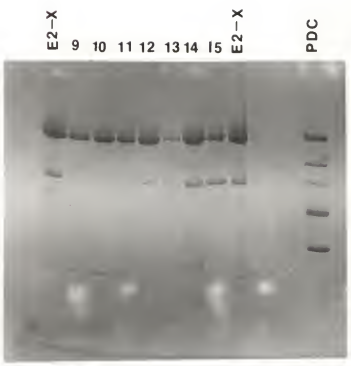
E2-X
9
10
11
12
13
14
15
E2-X
PDC

Figure 5. Typical E2 resolution profile. 1 mg E2-X-K_cK_b was incubated in 5.3 M urea, 0.35 M NaCl, and 2.0 mM DTT for 120 minutes. 0.4 ml fractions were collected and absorbance measured at 280 nm.

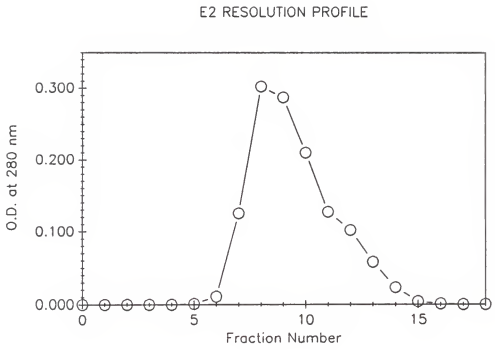
E2 RESOLUTION PROFILE
0.300
0.200
0.100
0.000
O.D. at 280 nm
0
5
10
15
Fraction Number

Figure 6. Subunits of the bovine kidney pyruvate dehydrogenase complex, the E2-X-K_cK_b subcomplex, and the E2 oligomer. The SDS-PAGE pattern is shown (left to right) for 6 µg of complex, 4 µg of E2-X-K_cK_b subcomplex and 3 µg of the E2 oligomer.


E2
E3
X
E1α
E1β

Figure 7. SDS-PAGE of the binding of the dihydrolipoyl dehydrogenase (E3) component. Pellet (P) consisted of 5 µl of the pellet fractions. Supernatant (S) consisted of 15 µl of the supernatant fractions. A pattern for 10 µg of the kidney pyruvate dehydrogenase complex is shown on the left side. Conditions for binding were described in the Experimental Procedures section.

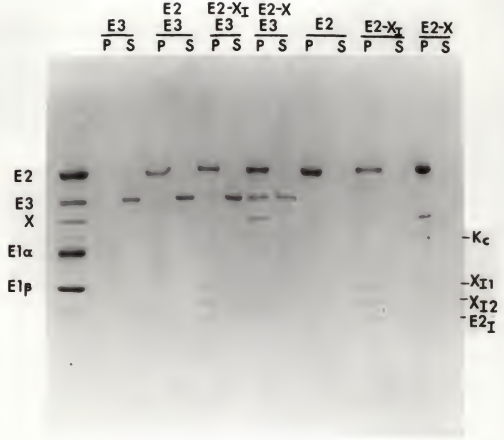
E3
P S
E2
E3
P S
E2-X_I
E3
P S
E2-X
E3
P S
E2
P S
E2-X_I
P S
E2-X
P S
E2
E3
X
E1α
E1β
K_c
X_I1
X_I2
E2_I

Figure 8. SDS-PAGE of the binding of the pyruvate dehydrogenase (E1) component. Labeling was as described in Figure 7. Conditions for binding were described in the Experimental Procedures section.

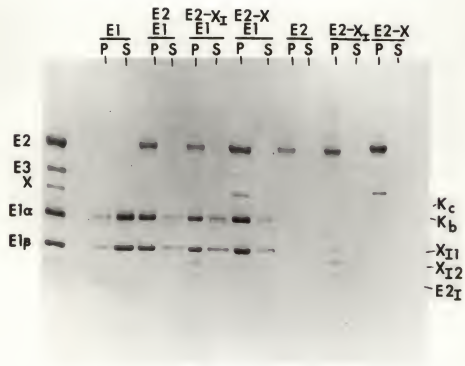
E1
E2
E1
E2-X_I
E1
E2-X
E1
E2
E2-X_I
E2-X
P S P S P S P S P S P S P S
E2
E3
X
E1α
E1β
K_c
K_b
X_I1
X_I2
E2_I

Table 1. Recovery of E2 oligomer. E2–X–K_cK_b subcomplex starting material and resolved E2 oligomer were assayed to determine transacetylation specific activity. E2 oligomer protein recovered was measured using the BCA assay (see Experimental Procedures). The total units were calculated of E2–X–K_cK_b starting material and of the E2 oligomer recovered.

E2 Source	Specific Activity	Recovery	Total units
E2–X–K (starting material)	7.7 μmoles/min/mg	1.0 mg	7.7 μmoles/min
E2 oligomer (after pelleting)	1.27 μmoles/min/mg	250 μg	0.32 μmoles/min

Table 2. Resolution procedure and results. A summary of the various conditions and their results as reported in RESULTS.

Incubation Mixture (50 mM Na_xPO_4 pH 7.5, 0.2 mM DTT) at 4° C	Column Buffer (50 mM Na_xPO_4 pH 7.5,0.5 mM DTT, 0.2 mM EDTA)	Results
5.0 M Urea 60 minutes	5.0 M Urea	Poor separation Good Transaceylation specific act. Low PDC specific act. Low yield (400 ug/lmg E2-X-K)
6.0 M Urea 60 minutes	6.0 M Urea	Good separation Average Transacetyl. specific act. Low PDC specific act. Low yeild (400 ug/lmg E2-X-K)
5.5 M Urea 60 minutes	5.5 M Urea	Good separation Good Transacetylation specific act. Low PDC specific act. Low yield (400 ug/lmg E2-X-K)
5.5 M Urea 0.35 M NaCl 60 minutes	5.5 M Urea 0.35 M NaCl	Good separation Good Transacetylation specific act. Low PDC specific act. Low yield (450 ug/lmg E2-X-K)
5.5 M Urea 0.35 M NaCl Pluronic F-68 (2 mg/ml) 60 minutes	5.5 M Urea 0.35 M Nacl Pluronic F-6 (2 mg/ml)	Poor separation Good Transacetylation specific act. Low PDC specific act. Good yield (700 ug/lmg E2-X-K)
5.3 M Urea 0.35 M NaCl 120 minute	5.3 M Urea 0.35 M NaCl Pluronic F-68 (0.2 mg/ml)	Complete separation Good Transacetylation specific act. Low PDC specific act. Good yield (700 ug/lmg E2-X-K)

Table 3. Comparison of the E2-X-K_cK_b subcomplex, E2-X_I subcomplex and E2 oligomer in the transacetylation reaction, reductive acetylation reaction, and the overall PDC reconstitution reaction.

E2 source	Trans-acetylation Reaction (mol/min/mg)	Overall PDC Reaction (mol/min/mg)	Reductive Acetylation (nmol[2-^{14}C] acetyl incorporated mg^{-1})	
			10s at 4°C	180s at 22°C
E2-X-K	3.6	18	9.2	13.8
E2-X_I	3.5	1.05	9.7	12.6
E2 Oligomer	4.0	0.73	4.1	6.9

Table 4. Microplate binding assays on E2 sources with different levels of protein X. E3 activity measured was based on implied protein concentrations bound to plate determined by BCA assay (see microplate binding assay protocol in Experimemtal procedures)[19].

E2 Source	µg ^{32}P-E1 bound per µg E2/X retained	Dihydrolipoyl Dehydrogenase Activity moles/min/µg E2/X retained
E2 oligomer	0.58	5.0
E2-X_I subcomplex	0.57	6.7
E2-X-K_cK_b subcomplex	0.62	13.5
X-E2 fraction	0.22	44.7

SEPARATION OF PROTEIN X FROM THE DIHYDROLIPOYL TRANSACETYLASE COMPONENT OF THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX AND THE STUDY OF PROTEIN X

by

Susan L. Powers-Greenwood

B.S., University of Nebraska, Lincoln, Nebraska, 1987

AN ABSTRACT OF A THESIS

submitted in partial fulfilment of the

requirements for the degree

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Department of Biochemsitry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1989

ABSTRACT

An oligomeric transacetylase (E2) component was produced free of protein X by resolution of the dihydrolipoyl transacteylase–protein X–kinase (E2–X–K_cK_b) subcomplex. The properties of this E2 oligomeric and a form of the subcomplex from which the lipoyl–bearing domain of protein X (X_L) was removed were studied. The subcomplex from which the X_L portion was removed and the oligomeric E2 component, while retaining other catalytic and binding properties of the native subcomplex, had greatly reduced capacities both to bind the dihyrolipoyl dehydrogenase (E3) component and to support the overall reaction of the complex (upon reconstitution with other components). These results indicate that protein X through its X_L domain, contributes to the binding of the E3 component and to the overall reaction of the pyruvate dehydrogenase complex.